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Original Article

N-acetylcysteine attenuates cyclosporin-induced nephrotoxicity in rats

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Abstract

Background. Cyclosporin (CsA) has played an important role in the improvement of solid-organ transplant patients and graft survival. However, nephrotoxicity due to CsA remains an important clinical challenge. The renal toxicity of CsA is attributed to reduced renal blood flow which leads to hypoxia–reoxygenation injury accompanied by excessive generation of oxygen-derived free radicals (ODFR). *N*-acetyl-L-cysteine (NAC) is a highly potent antioxidant that has been shown to reduce ODFR injury. In this study an attempt was made to assess the effect of NAC on CsA-induced lipid peroxidation and nephrotoxicity.

Methods. Adult Sprague–Dawley rats were treated orally with CsA (25 and 50 mg/kg) alone and in combination with different doses of NAC (10, 20 and 40 mg/kg) for a period of 3 weeks. Twenty-four hours after the last treatment, animals were sacrificed and blood was analysed for blood urea nitrogen (BUN) and serum creatinine (SCr), and kidney samples were analysed for lipid hydroperoxides, conjugated dienes and glutathione, and histopathological changes.

Results. Treatment of rats with CsA produced a significant increase in BUN and SCr level and histological abnormalities. CsA-induced impairment of renal toxicity was accompanied by significant increase in renal oxidative stress. NAC treatment significantly protected animals against CsA-induced structural and functional impairment of kidney.

Conclusions. CsA-induced nephrotoxicity was significantly attenuated by NAC. This study clearly suggests the role of oxidative stress in the pathogenesis of CsA-induced nephrotoxicity. Concomitant use of antioxidants such as NAC to minimize CsA-induced nephrotoxicity in humans warrant further studies.

Key words: cyclosporin; free radicals; glutathione; lipid peroxidation; *N*-acetylcysteine; nephrotoxicity

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Introduction

Cyclosporin (CsA) has improved patient and graft survival rates in solid-organ transplantation and has been increasingly applied with considerable clinical benefits in the treatment of autoimmune diseases. However, the therapeutic benefits of CsA are often limited by the occurrence of chronic nephrotoxicity which continues to be a major problem. The mechanism of CsA-induced nephrotoxicity is far from clear. Oral or parenteral administration of CsA causes increased renal vascular resistance and decreased renal blood flow as a result of the secondary release by resident or infiltrating cells of some autocoids [1]. Renal ischaemia, following impaired tissue perfusion, results in rapid breakdown of tissue ATP and a rise in its degradation products adenosine, inosine, and hypoxanthine. The enzymatic conversion of hypoxanthine to xanthine by xanthine oxidase generates superoxide radical (O^{-}_{2}) [2]. On the other hand it has been hypothesized that CsA-induced increase in the P-450 system may result in excessive free radical formation [3]. Another speculation linking the P-450 system to free radical mechanism for CsA toxicity is that CsA may uncouple cytochrome P-450, directing electron flow to molecular oxygen and thereby generating free radical. Recent studies clearly demonstrate that CsA-induced oxidative stress could play pivotal role in producing structural and functional impairment of kidney [3,4].

Of note is the widely accepted notion that the expression of tissue injury is determined not only by the nature of offending pathogen, but also by the quality of the host defence system. Since cells are equipped with various oxygen-derived free radical (ODFR) scavenging systems, it is plausible to speculate that it is when the amount of ODFR exceeds the capacity of cell or organ antioxidant system that the normal function is disrupted and tissue damage develops [2]. Recently vitamin E an effective antioxidant has been shown to protect rats against CsA-induced nephrotoxicity [5]. On the other hand endogenous sulph-hydryl compounds play an important role by acting as a metabolic buffer against oxidant species [6] and serve as a natural reservoir of reducing

power which can be quickly used in the cell as a defence against oxidative stress [6]. Glutathione (GSH) is a sulphur-containing nucleophilic substance found in high concentration in kidney. GSH plays a central role in protecting cells from ODFR injury and other activated toxic compounds [7]. *N*-acetyl-L-cysteine (NAC), a potent antioxidant by itself, may serve as a precursor for glutathione synthesis [8]. The stimulation of GSH synthesis following the administration of NAC results in a greater availability of GSH for detoxification of ODFR and other foreign substances [9]. NAC is routinely used in clinical practice in patients with acetaminophen overdose [10,11]. The protective effect of NAC has also been shown against ODFR-mediated gastric [12], hepatic [13], pulmonary [14], and cardiac [15] injuries. In consideration of the above, the present investigation was undertaken to study the effect of NAC on CsA-induced nephrotoxicity in rats.

Subjects and methods

Animals

Adult male Sprague–Dawley rats weighing 190–220 g were housed in a 12-h dark/light cycle animal facility with controlled temperature and humidity. Food and water were given *ad libitum* throughout the study.

Drugs

CsA (Sandoz Ltd, Basel, Switzerland) was dissolved in olive oil so as to give a final concentration of 25 mg/2 ml and 50 mg/2 ml. NAC (ICN, Aurora, Ohio) was dissolved in saline to a final concentration of 10 mg/2 ml, 20 mg/2 ml and 40 mg/2 ml. The drug solutions were freshly prepared and used on the same day.

Experimental groups

The rats were randomized and divided into eight groups of eight animals each. The rats in group 1 (control) were given olive oil, whereas the rats in groups 2, 3, 4 and 5 received CsA orally at a dose of 25 mg/kg body weight daily for 3 weeks. In addition to CsA, the animals in groups 3, 4 and 5 also received daily intraperitoneal (i.p.) injection of NAC in the doses of 10, 20 and 40 mg/kg respectively (30 min before CsA administration). Animals in group 6 received daily i.p. injection of NAC alone at a dose of 40 mg/kg. Animals in group 7 received CsA at a dose of 50 mg/kg orally whereas animals in group 8 received NAC at a dose of 40 mg/kg in addition to CsA at a dose of 50 mg/kg.Twenty-four hours after the last dose of CsA, the animals were sacrificed and blood was collected for various biochemical studies. The left kidney was immediately removed and stored at -70°C for the analysis of glutathione (GSH), lipid hydroperoxides (LPH), and conjugated dienes (CD). The right kidney was fixed in 10% neutral-buffered formalin for histological studies.

Biochemical analysis

All blood samples were allowed to clot at ambient temperature and centrifuged (2000 r.p.m. for 5 min) to harvest the serum. SCr and BUN were analysed using a Hitachi 717 (Boehringer, Mannheim, Germany) biochemical analyser.

Estimation of glutathione in kidney

Total glutathione was measured enzymatically by the method described by Mangino *et al.* [16]. About 50 mg of cold tissue was homogenized in 2 ml of 3% perchloric acid and 800 µl of the supernatant was neutralized with 2 drops of 10 N KOH. After the removal of the insoluble $KClO_4$, 200 µl of supernatant was assayed for total GSH. The following stock solutions were prepared on the day of use: solution I, 0.3 mM NADPH (Sigma); solution II, 6 mM 5,5′-dithio-bis (2-nitrobenzoic acid) (Sigma) and solution III, 50 U/ml glutathione reductase (Sigma). All three solutions were prepared with a stock buffer of 125 mM NaH_2PO_4 and 6.3 mM EDTA, pH 7.5. At the time of GSH assay, 700 µl solution I, 100 µl solution II, and 10 µl solution III were mixed in a quartz cuvette and placed in a dual beam UV-VIS spectrophotometer (Shimadzu UV 160) at 30°C. The enzymatic reaction was started by the addition of 200 µl of the sample, and the absorbance at 412 nM was monitored for 3 min. The slope of the change in absorbance was used to quantitate total GSH (Sigma). All GSH levels were normalized to 1 g wet tissue weight.

Determination of lipid hydroperoxides

Lipid hydroperoxides were measured using the iodometric method of Buege and Aust [17]. Kidney was homogenized in a tube containing 1 ml of Tris buffer (50 mM, pH 7.6) and 3 ml of 1.5% ethanolic pyrogallol. The homogenate was incubated at 70°C in a water bath for 5 min, and 150 µl of 10 M potassium hydroxide was added to each tube and further incubation (70°C) was done for 30 min. The mixture was cooled on an ice bath to room temperature and extracted with 2 ml of hexane. The organic layer was separated after centrifugation and the aqueous homogenate was further extracted with another 2 ml of hexane. The two extracts were combined and stored at -70°C for future analysis. The lipid extract was dissolved in 1 ml acetic acid : chloroform (3:2 v/v) mixture, and then 100 µl of 0.6 g/ml potassium iodide added. The tube was capped and kept in the dark for 5 min and then 3 ml of 0.5% cadmium acetate was added, mixed, and centrifuged at 3000 r.p.m. for 10 min. The absorbance of top layer was read at 353 nm using Shimadzu (Japan) Model 160A spectrophotometer. Lipid hydroperoxides were expressed as Abs_{353}/25 mg tissue and compared to a standard curve (cumene hydroperoxide).

Analysis of conjugated dienes

The level of conjugated dienes in the whole kidney was measured according to the method described by Handelman *et al.* [18]. Pre-minced renal tissue (25 mg) was homogenized with 1 ml of ethanol containing 1.2% pyrogallol at 4°C. The homogenate was saponified by adding 150 µl of 10 M potassium hydroxide and acidified to pH 3 using 1 M hydrochloric acid. The acidified homogenate was extracted with 3 ml of *n*-hexane. One millilitre aliquot of the *n*-hexane extract was evaporated under nitrogen and reconstituted with 2.5 ml of

cyclohexane. The level of conjugated dienes was determined by measuring the absorbance at 233 nm using a quartz cell.

Cyclosporin assay

The blood CsA assay was carried out 24 h after the last dose of CsA using commercially available kits (TDx/TDxFLx, Abbott Laboratories, Abbot Park, IL).

Histological study

After fixation, kidney was embedded in paraffin and sections of 3 μm thickness were stained with H & E and PAS. A semiquantitative assessment was performed to assess the extent of tissue injury. In brief, a minimum of 50 proximal tubules were examined for each specimen and the severity of lesion was graded from 0 to 3 according to the percentage of tubular involvement. Thus, 0 lesion represented no change; grade 1 lesion represented $<25\%$ of tubular damage (mild); grade 2 lesion represented 25–50% of tubular involvement (moderate); grade 3 lesion represented the involvement of $>50\%$ of the tubules (severe). The assessment was carried out in a blinded fashion.

Statistical analysis

Results are expressed as mean $\pm$ SEM. Data from different groups were compared using Dunnett's multiple range test. $P<0.05$ was considered significant.

Results

Changes in body weight, food and water intake, and kidney weights

CsA treatment produced a significant decrease in body weight gain as compared with control animals (Figure 1). Treatment with NAC did not affect the weight gain pattern in control or CsA-treated rats. Treatment of rats with NAC alone did not produce any significant change in food or water intake of animals (Table 1). However, a significant decrease in food and water intake of rats treated with CsA alone or CsA plus NAC was noticed as compared to control (Table 1). There was no significant change in the water content of kidney in any treatment group (Table 1).

Effect of CsA and NAC on BUN, SCr and CsA level

There was a significant increase in BUN and SCr level in CsA-treated rats as compared to the control group (Table 2). Treatment of rats with NAC (alone) did not cause any significant change in BUN or SCr level as compared to control group; however, NAC attenuated CsA-induced increase in BUN and SCr levels in a dose-dependent manner (Table 2). Concomitant treatment of rats with NAC dose-dependently decreased the blood level of CsA. However, these changes were not found to be statistically significant (Table 2).

Renal LPH, CD, and GSH levels

Treatment of animals with CsA alone produced a significant increase in renal LPH and CD levels and decrease in GSH level. Cotreatment with NAC significantly reversed CsA-induced changes in the ODFR indices (Table 3).

Histological studies

The kidneys of the rats treated with NAC alone showed no histopathological abnormality. The treatment of rats with CsA produced moderate to severe vacuolations and necrosis (Figure 2, Table 4) in the straight and convoluted segments of proximal tubules. The distribution was patchy, involving single or multiple tubules in any given area. CsA also produced mild to moderate interstitial fibrosis and parietal cell hyperplasia (Table 4). Occasionally, mild focal glomerular sclerosis, interstitial fibrosis, juxtaglomerular hypertrophy, and arteriolopathy was also observed in the CsA-treated group. Concomitant treatment of animals with NAC significantly attenuated CsA-induced histopathological changes (Figure 3, Table 4).

Discussion

Treatment of rats with CsA for a period of 3 weeks resulted in a significant increase in BUN and SCr levels (Table 2), suggesting a significant functional impairment of kidneys. Our results are in agreement with earlier investigators, who reported significant alteration in BUN and SCr in patients and experimental animals following treatment with CsA [1]. Our histopathological studies also showed significant structural changes in kidneys including tubular vacuolations and necrosis, interstitial fibrosis, and parietal cell hyperplasia following treatment with CsA (Table 4).

Similar histopathological changes including interstitial fibrosis and proximal and/or distal tubular damage have been reported following treatment with CsA for extrarenal diseases [1]. Concomitant treatment of rats with NAC significantly attenuated the CsA-induced structural and functional changes in kidney. The precise mechanism of NAC-induced protection against CsA nephrotoxicity is far from clear. Both acute and chronic administration of CsA have been shown to increase significantly renal vascular resistance, hypoperfusion, and ischaemia [1]. Many substances such as angiotensin, nitric oxide (NO), and thromboxane/prostacyclin are regarded as possible mediators of CsA-induced vasoconstriction [19–21]. However, recent studies suggest an important role of endothelin in CsA-induced increase in vascular resistance [22,23]. Increased production of endothelin by vascular smooth-muscle cells in the medium containing CsA has been reported [24]. Furthermore, endothelin receptor antagonist BQ 123 has been shown to ameliorate CsA-induced vascular resistance [25]. Endothelin has also been shown to affect renin—angiotensin system [26] and to inhibit NO and prostaglandin production leading to vasoconstriction [27].

On the other hand, NAC has been shown to decrease vascular resistance and enhance tissue perfusion [28].

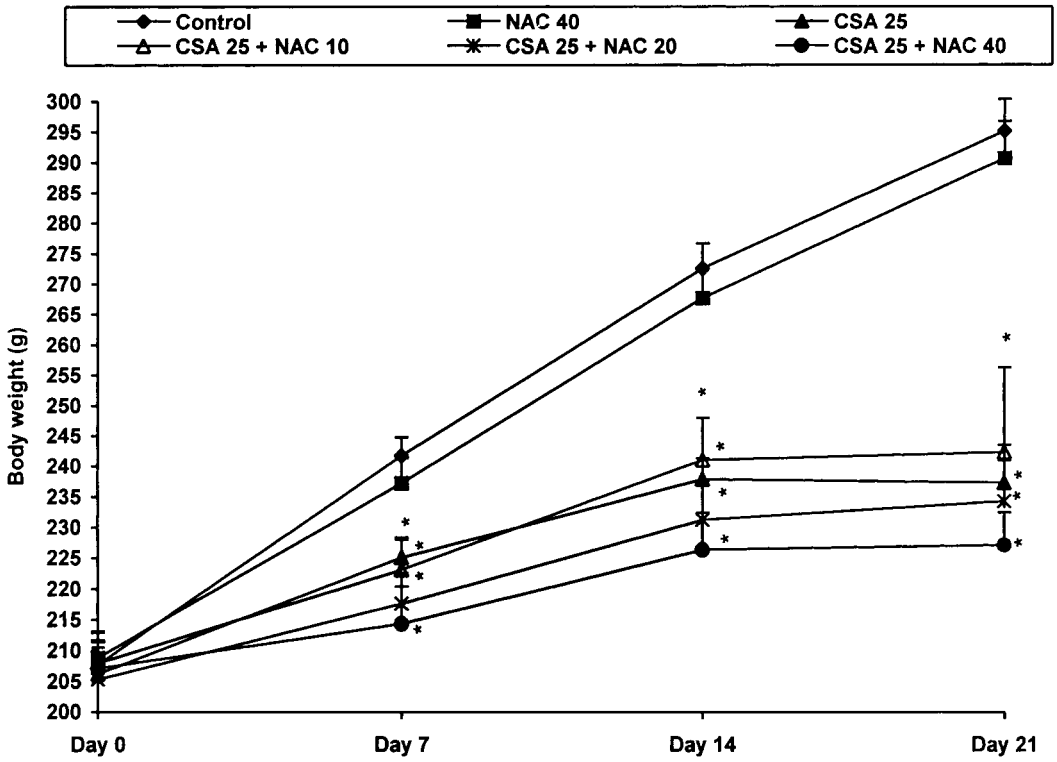

Fig. 1. Changes in body weight of animals in different treatment groups. The animals in different treatement groups gained weight throughout the period of study. However, the rate of weight gain was higher in control group as compared to any other treatment group (*$P<0.01$ *vs* control).

Table 1. Mean food intake (g/100 g body weight), water intake (ml/100 g body weight), and kidney weight (% water content) in different treatment groups

No.	Group	Food	Water	Kidney weight
1	Control	7.06 ± 0.19^{a}	18.13 ± 0.26	76.40 ± 0.22
2	NAC 40	6.92 ± 0.15	18.85 ± 0.13	76.33 ± 0.47
3	CsA 25	4.11 ± 0.38^{c}	15.08 ± 0.83^{b}	$76.6\ \pm0.33$
4	CsA 25 + NAC 10	4.71 ± 0.10^{c}	14.54 ± 0.10^{c}	–
5	CsA 25 + NAC 20	4.39 ± 0.27^{c}	16.85 ± 0.33^{b}	–
6	CsA 25 + NAC 40	4.20 ± 0.16^{c}	15.41 ± 0.14^{c}	76.10 ± 0.27
7	CsA 50	3.85 ± 0.10^{c}	15.06 ± 0.42^{c}	76.06 ± 0.28
8	CsA 50 + NAC 40	4.07 ± 0.17^{c}	14.98 ± 0.50^{c}	76.23 ± 0.24

[a]Values are mean $\pm$ SEM; [b]$P<0.05$; [c]$P<0.01$ *vs* control.

Table 2. Effect of NAC on CsA-induced changes in BUN, SCR, and CsA level

Group	BUN (mmol/l)	SCR (mmol/l)	CsA (nmol/l)
Control	5.40 ± 0.15^{a}	50.80 ± 0.70	–
NAC 40	5.30 ± 0.10	50.30 ± 0.40	–
CsA 25	10.90 ± 0.37^{b}	59.13 ± 1.10^{b}	2321.85 ± 306.65
CsA 25 + NAC 10	9.08 ± 0.34^{c}	54.14 ± 0.74^{c}	2052.83 ± 263.86
CsA 25 + NAC 20	8.16 ± 1.10^{c}	54.80 ± 0.70^{c}	1995.35 ± 249.30
CsA 25 + NAC 40	7.02 ± 0.58^{d}	51.50 ± 1.80^{c}	1736.58 ± 197.69
CsA 50	11.42 ± 0.60^{b}	60.80 ± 1.56	3604.64 ± 190.64
CsA 50 + NAC 40	8.82 ± 0.10^{e}	56.60 ± 1.60	3192.49 ± 225.5

[a]Values are mean $\pm$ SEM; [b]$P<0.01$ *vs* control; [c]$P<0.05$; [d]$P<0.01$ *vs* CsA alone group.

Table 3. Effect of NAC on CsA-induced changes in renal lipid hydroperoxide, conjugated dienes, and glutathion levels

Group	Lipid hydroperoxides (Abs_{353}/25 mg tissue)	Conjugated dienes (Abs_{353}/25 mg tissue)	Glutathione (nmol/g)
Control	0.142 ± 0.003^{a}	0.098 ± 0.003	248.37 ± 6.5
NAC 40 alone	0.143 ± 0.003	0.108 ± 0.003	486.28 ± 6.5
CsA 25	0.177 ± 0.009^{b}	0.135 ± 0.005^{c}	58.8 ± 2.4^{c}
CsA 25 + NAC 10	0.153 ± 0.002^{d}	0.106 ± 0.005^{e}	382.28 ± 8.4^{e}
CsA 25 + NAC 20	0.153 ± 0.003^{d}	0.099 ± 0.006^{e}	415.75 ± 6.5^{e}
Csa 25 + NAC 40	0.156 ± 0.002^{d}	0.100 ± 0.001^{d}	473.85 ± 8.1^{e}

[a]Values are mean ± SEM; [b]$P < 0.05$; [c]$P < 0.01$ *vs* control' [d]$P < 0.05$; [e]$P < 0.001$ *vs* CsA alone group.

Table 4. Histopathological changes in different treatment groups

	Injury score			
Group	Tubular vacuolation	Tubular necrosis	Interstitial fibrosis	Parietal cell hyperplasia
Control	0	0	0	0
NAC 40 alone	0	0	0	0
CsA 25	2.71 ± 0.18^{a}	2 ± 0.21	1.85 ± 0.26	1.71 ± 0.18
CsA 25 + NAC 10	1.28 ± 0.18^{c}	1.14 ± 0.14^{b}	1.14 ± 0.14^{b}	0.85 ± 0.26^{b}
CsA 25 + NAC 20	1.42 ± 0.20^{c}	1 ± 0.21^{b}	1.14 ± 0.14^{b}	1.14 ± 0.14^{b}
CsA 25 + NAC 40	1.14 ± 0.14^{c}	1 ± 0.21^{b}	0.85 ± 0.14^{b}	1 ± 0.21^{b}

Higher scores indicate severe injury. [a]Values are mean ± SEM; [b]$P < 0.05$; [c]$P < 0.01$ *vs* CsA alone group.

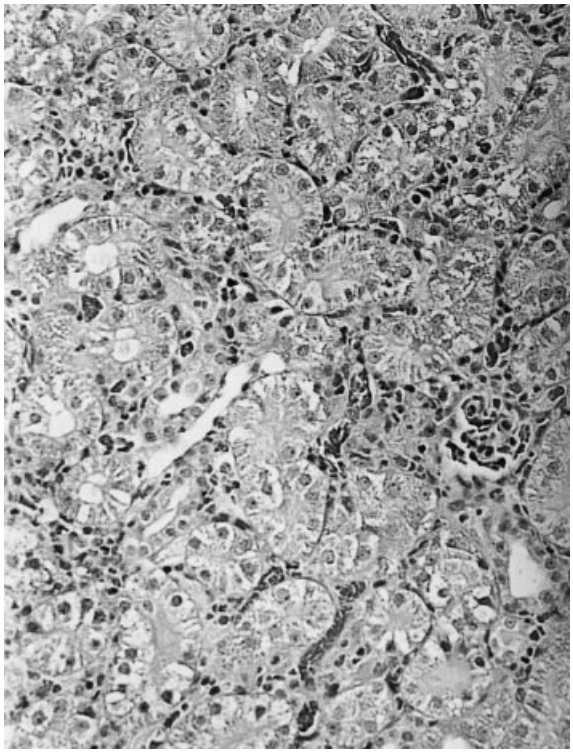

Fig. 2. Photomicrograph from CsA-treated kidney (CsA 25 mg/kg, 3 weeks) showing severe vacuolations and necrosis (H&E × 500).

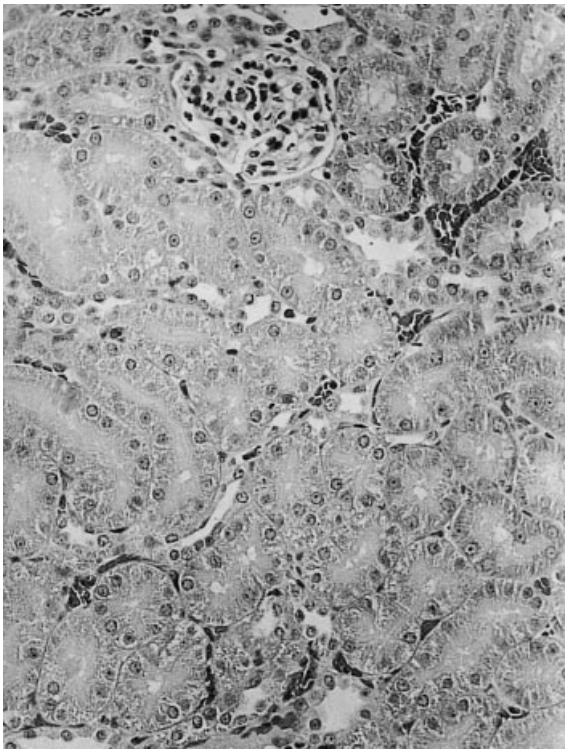

Fig. 3. Photomicrograph of kidney from CsA-treated (25 mg/kg) plus NAC-treated (40 mg/kg) group. Cortical tubules show mild necrosis and vacuolations (H&E × 500).

The vasodilating effects of NAC may be attributed to its direct relaxing action on vascular smooth muscle and its ability to inhibit angiotensin-converting enzyme [29]. NAC stimulates the release of NO, which in turn may decrease CsA-induced vascular resistance [30]. Recently NAC has been shown to indirectly modulate the action of endothelin on microvessels [31]. Furthermore, CsA-induced nephrotoxicity is associated with accumulation of cellular caclium, and

calcium-channel blockers have been shown to attenuate CsA-induced renal damage [32]. Sulph-hydryl compounds including NAC have been shown to block calcium channels and maintain calcium homeostasis [33].

The treatment of rats with CsA produced a significant increase in renal CD and LPH and decrease in glutathione level (Table 3), suggesting the role of oxidative stress in CsA nephrotoxicity. Treatment with CsA has been shown to increase $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot -}$ radicals production [34]. Oxygen radicals are considered as important modulators of renal blood flow and glomerular filtration rate [35]. Lipid peroxidation begins as a result of ODFR-induced abstraction of hydrogen from a polyunsaturated fatty acid of cellular membrane forming a lipid radical, the rearrangement of which results in formation of relatively stable conjugated dienes. The lipid-peroxy radical results from subsequent interaction with molecular oxygen and hydrogen abstraction from an adjacent lipid hydroperoxide which is accompanied by cellular degeneration. An efficient endogenous antioxidant defence system operates to combat free radicals. The main detoxifying system for lipid peroxides is GSH [9]. The decrease in GSH following CsA observed in this study is supported by earlier investigation [36]. NAC is a potent antioxidant which may scavenge a wide variety of ODFR including $O_2^{\cdot -}$, H_2O_2 and $O^{\cdot}H^-$ radicals [37–39]. Barth *et al.* [40] showed the protective effect of NAC against CsA-induced lipid peroxidation in liver microsomes.

Treatment of rats with NAC significantly attenuated CsA-induced depletion of GSH (Table 3). GSH dependent mechanisms play a vital role in the protection of cells against oxidative stress and detoxification of xenobiotics including CsA [9,41]. Besides its antioxidant activity, GSH retains cellular metabolic functions and integrity by stabilizing cell membrane. Though GSH supplementation has been shown to offer protection against ischaemic injury [42], the major disadvantage is that GSH does not pass through the cell membrane and its action may be a function of its extracellular level only [9]. On the other hand NAC, a precursor of GSH, readily passes through the cell membrane thereby resulting in quick replenishment of intracellular GSH. It has been shown that sulph-hydryl compounds which pass intracellularly are more effective than those that remain extracellular [43]. Thus, the ability of NAC to increase intracellular and extracellular GSH might be a crucial factor in protecting renal tissue in CsA-induced oxidative damage.

Concomitant treatment of NAC along with CsA produced a mild (statistically non-significant) decrease in blood CsA level. Furthermore, some earlier studies have shown a direct adduct formation between NAC and various xenobiotics [10]. Thus the alteration in bioavailability of CsA by NAC may to some extent contribute to the protective effect of the latter drug. Finally, the data presented here suggest that concomitant use of antioxidants such as NAC might be useful in reducing CsA-mediated nephrotoxicity.

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